

***Plagiorchis elegans*: Requirements for Metacercarial Development to Infectivity, and Conditions Required for Excystment**

CARL A. LOWENBERGER AND MANFRED E. RAU¹

Institute of Parasitology, Macdonald Campus of McGill University, 21,111 Lakeshore Rd.,
Ste Anne de Bellevue, Québec, H9X 1C0, Canada

ABSTRACT: The temperatures and times required for the development of *Plagiorchis elegans* (Digenea: Plagiorchiidae) metacercariae from encystment to infectivity were studied in an experimental host, *Aedes aegypti* (Diptera: Culicidae). The ability of metacercariae to excyst was evaluated in vitro and was equated with infectivity to the definitive host. Development of metacercariae to infectivity followed a sigmoidal curve at temperatures between 15 and 30°C. Rates of development increased significantly with temperature. At 15°C metacercariae first excysted 72 hr postinfection (PI); at 30°C this occurred as early as 12 hr PI. Excystment reached 80% after 132 hr at 15°C and 60 hr at 30°C. A minimum of 8 hr of contact with the host was required for subsequent development to infectivity in vitro. Excystment of infective metacercariae was temperature dependent. Less than 6% of metacercariae excysted at $\leq 30^\circ\text{C}$, whereas $\geq 80\%$ excysted at $\geq 37^\circ\text{C}$. Such temperature requirements may explain in part why adult *P. elegans* are principally parasites of homeothermic animals.

KEY WORDS: *Plagiorchis elegans*, Digenea, Trematoda, metacercaria, *Aedes aegypti*, excystment, in vitro.

Cercariae of *Plagiorchis elegans* (Rudolphi, 1802) actively penetrate their insect intermediate hosts and then undergo morphological and physiological changes from free-swimming cercariae to encysted, relatively inactive metacercariae (Lackie, 1975). A variety of environmental factors may affect the subsequent development of the parasite toward infectivity to the vertebrate definitive host. Metacercariae must be adapted to survive intermediate host defense mechanisms as well as the digestive system of the definitive host. In the intestinal tract of the definitive host, metacercariae must respond to 1 or many of a wide range of stimuli, excyst, and attach to the gut lining (Lackie, 1975). This requires that the parasite has reached a stage of development that allows it to receive and respond quickly to such stimuli.

The present study determined the temperature and time requirements for development of *P. elegans* metacercariae to infectivity in an experimental insect host, *Aedes aegypti* (L.) and also determined the conditions and temperature requirements for excystment of infective metacercariae in vitro.

Plagiorchis elegans

The behavior and development of the various stages of *P. elegans* have been described by Macy (1960), Blankespoor (1977), and Genov and Samnaliev (1984). Cercariae of species of the ge-

nus *Plagiorchis* are released from the molluscan first intermediate hosts and penetrate a number of aquatic insects as well as crustaceans (Williams, 1963). Cercariae attach to the host cuticle and penetrate by means of a stylet and histolytic enzymes (Bock, 1984; Taft, 1990) and then encyst as metacercariae in the hemocoel. The time required for metacercariae to reach infectivity is reported as 4–5 days for *Plagiorchis noblei* (Blankespoor, 1974) and 3 days for *P. elegans* (Genov and Samnaliev, 1984). Metacercariae excyst and transform into adults in the intestine of the definitive host.

Materials and Methods

Groups of 200 fourth instar *A. aegypti* were maintained in plastic containers containing 300 ml tap water and fed ground Tetramin® fish food ad libitum. A 16:8 light:dark regime was maintained in each temperature-regulated incubator. Infected snails, *Stagnicola elodes* (Say), were placed in the dark to induce cercarial emergence. Eight hours later approximately 1,000 cercariae were introduced to each container of mosquitoes, which were maintained at 15, 20, 25, 30, or 35°C. Twenty minutes postexposure the mosquitoes were transferred to containers of clean water to prevent subsequent infection and maintained at the respective temperatures.

At 12-hr intervals postexposure, 20 metacercariae were dissected from each group of larvae, placed in an artificial excystment medium, MBEM (0.015 M NaHCO₃, 0.015 M NaCl, 0.5 g/l bile salts [50:50 Na cholate:Na deoxycholate] (Sigma B-8756), pH 7.5), modified after Bock (1986), and incubated at 37°C. The number of successfully excysted metacercariae was recorded 2 hr following incubation. This was repeated 5 times at each temperature. For the purpose of this study,

¹ To whom reprint requests should be addressed.

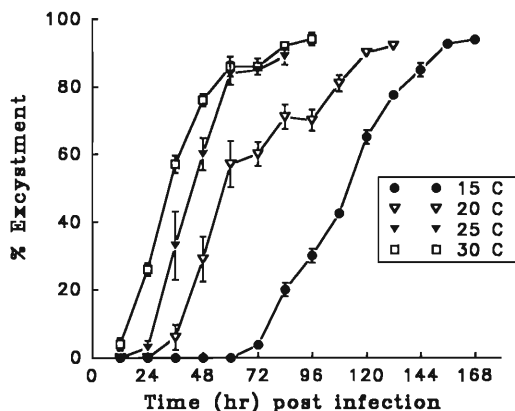


Figure 1. Excystment of *Plagiorchis elegans* metacercariae removed from the insect host at various times postinfection. Data are presented as mean % excystment \pm SE.

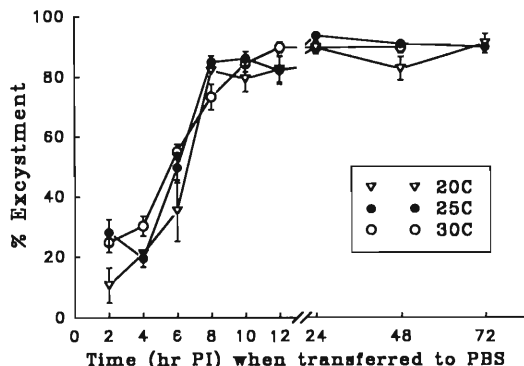


Figure 2. Excystment of *Plagiorchis elegans* metacercariae removed from the mosquito host at various times postinfection and maintained in PBS until they reached the age required for 80% excystment as determined from Figure 1. Data are presented as mean % excystment \pm SE.

a successful excystment was considered to produce a mobile juvenile digenean. Those partially emerged or still bound to the cyst wall were considered nonviable.

In order to test the *in vivo* contribution of the host to the development of the parasite, mosquitoes were infected as above and maintained at 20, 25, and 30°C. Metacercariae were dissected from mosquitoes at 2-hr intervals postinfection for the first 12 hr, and subsequently at 12-hr intervals. Groups of 20 such metacercariae were transferred to phosphate-buffered saline (PBS) and maintained at the same temperature as the mosquito host from which they had been removed. Media were replaced daily. This procedure was repeated 5 times at each temperature. At the time when *in vivo* incubation at the particular temperature would consistently provide >80% excystment of metacercariae *in vitro* (as determined by the first experiment), metacercariae were transferred from PBS to MBEM at 37°C. All metacercariae maintained at the same temperature were excysted at the same age postinfection. The number of metacercariae successfully excysting within 2 hr following the transfer was recorded.

To test the range of temperatures that will induce excystment of metacercariae, mosquitoes were infected as above, and the metacercariae allowed 6 days at 20°C to reach infectivity. Groups of 20 infective metacercariae were removed from the hosts and were placed in MBEM at temperatures ranging from 15 to 45°C. The number of successful excystments was recorded at 15-min intervals for 2 hr. This was repeated 5 times at each temperature.

The effect of temperature alone on excystment was studied by placing groups of 20 metacercariae in PBS or RPMI medium at 37°C for 2 hr. Unexcysted metacercariae were subsequently placed in MBEM at 20°C for 2 hr. Those that still had not excysted were then incubated in MBEM at 37°C for 2 hr.

Statistical analysis was done using SYSTAT 5.0 software using the Mann-Whitney *U*-test, Tukey's multiple comparison tests, and Student's *t*-test. The level of significance (α) was set at 0.05.

Results

The effect of temperature on metacercarial development within the insect host was significant. There was an inverse relationship between temperature and time required to reach infectivity. The first successful excystment at 15°C occurred after 72 hr, compared to 36, 24, and 12 hr at 20, 25, and 30°C, respectively. Levels of excystment reached 80% after 132, 108, 60, and 60 hr at 15, 20, 25, and 30°C, respectively. The development of metacercariae at each temperature, as measured by the ability to excyst in MBEM, followed a sigmoidal curve (Fig. 1).

Metacercariae that received ≥ 8 hr of host contact showed no significant difference in their rate of development to infectivity compared with metacercariae maintained entirely in the larval hosts. However, regardless of temperature, a minimum of 8 hr of host-parasite contact was required to ensure development to 80% excystment levels (Fig. 2). Some metacercariae with fewer than 8 hr of host contact did excyst successfully, but levels of excystment were significantly lower (Mann-Whitney *U*-test, $P < 0.05$). Host-parasite contact in excess of 8 hr did not significantly increase levels of excystment (Mann-Whitney *U*-test, $P > 0.05$).

The excystment of infective metacercariae was temperature dependent. Less than 6% of the metacercariae excysted at temperatures $\leq 30^\circ\text{C}$. There was no significant difference (Tukey HSD, $P > 0.05$) between levels of excystment at temperatures $\geq 37^\circ\text{C}$ (Fig. 3). Levels of excystment

at 35°C were intermediate and significantly different from those at ≤30 and ≥37°C (Tukey HSD, $P < 0.05$) (Fig. 3).

Elevated temperatures alone were insufficient in inducing excystment. When infective metacercariae were placed in PBS or RPMI at temperatures between 37 and 40°C, <5% of the metacercariae excysted. Such unexcysted metacercariae failed to excyst when transferred to MBEM at 20°C. However, subsequent transfer to MBEM at 37°C, resulted in >80% successful excystment within 2 hr (Table 1). Significant differences ($P < 0.05$) existed between groups containing MBEM at 37°C and all other groups (Student's t -test, $P < 0.05$).

Discussion

Whether metacercarial development is directly affected by temperature or is mediated through the physiology of the insect host was not addressed in this study. Reduced temperatures in the presence of an adequate food supply slow the development of mosquito larvae (Christophers, 1960) and thus may also affect developing parasites. The developmental curves of metacercariae in mosquitoes were sigmoidal and parallel at the various temperatures (Fig. 1), differing only in the lag phase.

The contribution of the host per se to metacercarial development is unknown. After only 8 hr within the host, metacercariae are able to develop to infectivity in nonnutritive PBS. Although overall development of metacercariae is temperature dependent (Fig. 1), there is a temperature-independent phase of metacercarial development; the first 8 hr of host contact are required by all metacercariae independent of ambient temperature (Fig. 2). There are several explanations for this, all related to the uptake of essential nutrients from the host: (1) The metacercariae may have exhausted reserves during

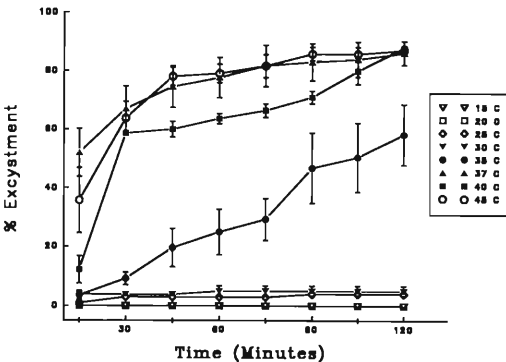


Figure 3. Excystment of infective *Plagiorchis elegans* metacercariae following 2 hr incubation in excystment medium at various temperatures. Data are presented as mean % excystment ± SE.

penetration and encystment and may require 8 hr to restore nutrient levels required for subsequent development. (2) Uptake of specific compounds may require 8 hr to be completed, or metacercariae may take up nutrients only following the completion of cyst wall formation. (3) The cyst walls produced by the parasite or the third cyst layer of host origin may impede the rapid influx of nutrients and thus impair parasite development. (4) Encapsulation and melanization of the cyst by the insect host may interfere with nutrient uptake. All of the above suggest a nutrient dependency on the host during the first 8 hr following encystment.

Some successful excystment occurs when cysts are removed from the insects earlier than 8 hr postinfection. However, excystment of these metacercariae is characteristically and significantly less than those that have received >8 hr of host contact (Fig. 2). Smyth and Halton (1983) state that metacercariae can absorb nutrients during the process of growth and differentiation. What nutrients cross the cyst wall, either actively

Table 1. Excystment of infective *Plagiorchis elegans* metacercariae exposed for 2 hr to each of a sequence of media and temperatures.

Sequence of conditions	% Excystment*	Sequence of conditions	% Excystment	Sequence of conditions	% Excystment
PBS 37°C	2.0 ± 1.2	RPMI 37°C	1.0 ± 1.0		
↓		↓			
MBEM 20°C	2.0 ± 1.2	MBEM 20°C	1.6 ± 0.3	MBEM 20°C	1.2 ± 0.2
↓		↓		↓	
MBEM 37°C	84.0 ± 2.9	MBEM 37°C	85.2 ± 2.1	MBEM 37°C	86.2 ± 4.1

* Cumulative mean % excystment ± SE.

or passively, is not known. The completion of the 2-layered cyst wall may limit the influx of some compounds and reduce uptake (Bock, 1988), and Taft (1990) suggests the third cyst layer of host origin may have a similar effect. The speed with which these layers, particularly the third, are deposited may vary with the location of the cyst and the occurrence of other host-mediated factors (Taft, 1990). When the deposition of this third layer of cyst wall is delayed or absent, metacercariae may obtain nutrients more effectively and may reach infectivity with <8 hr of host contact.

Within the range of temperatures used, metacercariae showed a distinct pattern of development, the most visible of which is an increase in the size and optical density of the excretory vesicle. The cyst walls may restrict the elimination of excretory products. Alternatively, by retaining these products, the parasite may avoid stimulating host defense mechanisms. The size and conspicuousness of this vesicle can be used as an indicator of the age and infectivity of metacercariae.

Excystment of metacercariae may be passive or active, and parasites that are ingested by their hosts as inactive stages may play an active role in their own excystment (Lackie, 1975). The metacercariae of species of *Plagiorchis* excyst intrinsically. Bock (1986, 1989) found that metacercariae of *Plagiorchis* sp. 1 became very active when exposed to an artificial excystment medium. The juveniles egested stored cecal fluid against the inner walls of the cyst and actively emerged through this area. This "explosive expulsion" of juveniles has been reported by other authors (Howell, 1970; Bass and LeFlore, 1984). The internal pressure of the metacercariae aids in rapid excystment and allows early attachment by the parasite to the intestine (Bock, 1989). Bile and bile salts (a component of MBEM) stimulate muscular activity in a number of species (Lackie, 1975). This implies that the metacercariae are mature and are ready to contribute actively to their liberation under appropriate conditions. Some metacercariae excyst to produce immobile, nonviable juveniles that differ morphologically from normal individuals. This occurs most commonly when metacercariae normally approach infectivity and is characterized by a poorly developed excretory vesicle. Although such metacercariae may not have developed fully, they nevertheless respond to the excystment stimulus.

The ability to excyst may precede the ability to survive in a postexcystment environment.

In many digenean species, excystment is temperature dependent and may be inhibited by incubation at inappropriate temperatures (Dixon, 1966). Similarly, *P. elegans* metacercariae showed essentially no excystment in MBEM at temperatures <35°C. However, when transferred to MBEM at 37°C, >80% of the worms excysted within 2 hr. High temperature alone did not induce excystment. There were significant differences in the levels of excystment in those groups that included exposure to MBEM at 37°C and all other groups (Table 1). A suitable temperature and medium must be present simultaneously to ensure significant levels of excystment. Threshold temperatures for excystment exist for many digeneans, and metacercariae will not excyst in vitro unless the temperature approximates that of the definitive host (Thompson and Halton, 1982; Asanji and Williams, 1975). Temperature differences of as little as 4°C may influence excystment levels (Fried and Huffman, 1982).

Bock (1986) concluded that although pretreatment by passage through the stomach was not required for excystment, contact with gastric juices enhanced the effects of bile. Because *Plagiorchis* sp. 1 excyst at temperatures as low as 21°C, Bock (1986) chose the speed of excystment as a criterion of successful excystment. In contrast, *P. elegans* metacercariae did not excyst at temperatures <30°C.

The present study defines the temperature-time relationship for the development of metacercariae of *P. elegans* to infectivity within an insect intermediate host. We have shown a temperature-independent obligatory period of host-parasite contact followed by a temperature-dependent development period to obtain infective metacercariae. The physiological interactions between intermediate host and parasite, including nutrient uptake, remain unclear. Active excystment of infective metacercariae requires both appropriate temperatures ($\geq 37^\circ\text{C}$) and an activating stimulus.

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